Chapter 7 | Prognostic impact of germinal center–associated proteins and chromosomal breakpoints in poor-risk diffuse large B-cell lymphoma

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BACKGROUND

Outcome of diffuse large B-cell lymphoma (DLBCL) with a germinal center B-cell (GCB) expression profile is superior to that of non-GCB DLBCL. This conclusion is mainly derived from patients with mixed international prognostic index (IPI) risk profiles treated with CHOP-like therapy (cyclophosphamide, doxorubicin, vincristine, and prednisone). We wondered whether the prognostic impact of the expression profile would hold out in a homogeneous cohort of poor-risk DLBCL patients treated with high-dose sequential therapy (HDT) and autologous stem-cell transplantation (ASCT) as first-line therapy.

PATIENTS AND METHODS

DLBCL from 66 newly diagnosed poor-risk patients, treated in two sequential prospective Dutch Hemato-Oncology Association (HOVON) trials, were studied retrospectively for expression of CD10, bcl6, MUM1/IRF4, bcl2, Ki-67, and CD21+ follicular dendritic cells (FDC) by immunohistochemistry, and for the breakpoints of *BCL2*, *BCL6*, and *MYC* by fluorescent in situ hybridization (FISH). Lymphomas with any follicular component were excluded.

RESULTS

A GCB immunophenotype profile was found in 58% and non-GCB immunophenotype profile in 42% of the tumors. Clinical characteristics of both groups were similar. Complete response (CR) rate was higher in patients with CD10+ tumors (58% v 30%; p=0.03). A GCB immunophenotype profile, its constituting markers CD10 more than 30% and MUM1 less than 70%, and bcl2 less than 10% were each associated with a better overall survival (OS). FDC networks, equally present in GCB and non-GCB tumors, had superior CR (73% v 31%; p=0.01), but disease-free survival rates were lower and there was no difference in OS rates. None of the breakpoints had a prognostic impact on outcome.

CONCLUSIONS

Also in patients with poor-risk DLBCL treated with HDT and ASCT, the GCB immunophenotype and bcl2 expression retained a major impact on survival.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL), the most common type of aggressive non-Hodgkin's Lymphoma (NHL), exhibits distinct biologic and clinical heterogeneity.[1] Currently, the international prognostic index (IPI) is still the most important tool to predict response to treatment for aggressive NHL and to classify patients into subgroups with distinctly different prognoses.[2] However, even within these IPI riskgroups a substantial variability in outcome has been observed. Thus, finding new tools to better classify DLBCL patients in different prognostic subgroups is important.

Many studies have focused on the significance of the intrinsic characteristics of the tumors, eg, protein or gene expression and chromosomal breakpoints, however, the clinical relevance of many of these markers is inconsistent. Moreover, few markers retain sufficient prognostic significance individually after adjustment for the overriding prognostic impact of the IPI risk score.[3,4]

Gene expression profiling offers prognostic value in DLBCL independently of IPI. Tumors with profiles closely resembling that of germinal center B-cells (GCB) have a better outcome than those with profiles resembling activated peripheral B-cells (ABC).[5-7] However, not all studies could confirm a prognostic value.[8,9] Furthermore, this analysis is not easily incorporated in routine practice as it depends on the availability of frozen tissue and sophisticated laboratory and statistical methods.

Based on gene expression studies, Hans et al[10] developed an algorithm to discriminate GCB from non-GCB type DLBCL, based on CD10, bcl6, and MUM1/IRF4 expression measured by immunohistochemistry (IHC). Outcome of DLBCL with a GCB protein expression profile was superior to non-GCB tumors and concurred with gene expression profiles as determined by micro-array analyses.[10] Controversial findings have been reported using the same or slightly different IHC algorithms,[11-15] which might be caused by differences in clinical characteristics, variations in treatment, IHC techniques, or cutoff levels for scoring. Moreover, most data published so far have been derived from patients treated with standard (ie, CHOP-like; cyclophosphamide, doxorubicin, vincristine, and prednisone) firstline treatment. Whether up-front high-dose sequential therapy (HDT) and autologous stem-cell transplantation (ASCT) as first-line treatment might overcome the poor prognostic features conferred by cell of origin is still unknown.

We recently reported on two sequential prospective trials of the Dutch Belgian Hemato-Oncology Cooperative Group (HOVON), HO27 and HO40, investigating HDT and ASCT as first-line treatment in patients with poor-risk advanced-stage aggressive NHL with age adjusted IPI scores of 2 to 3.[16] In this article, we report the results of a retrospective analysis addressing the prognostic significance of individual protein expression, GCB versus non-GCB immunophenotype, and chromosomal breakpoints in the DLBCL patients of both studies.

PATIENTS AND METHODS

Patient selection

Patients were included in either one of two consecutive HOVON trials, HO27 and HO40, for previously untreated poor-risk aggressive NHL.[16] In summary, patients had aggressive NHL, were age 18 to 65 years, had WHO performance status 0 to 2, had Ann Arbor stage III or IV disease, and had serum lactate dehydrogenase (LDH) of at least 1.5 times the upper limit of normal (ULN). Treatment consisted of high-dose sequential chemotherapy followed by HDT and ASCT (trial HO27) or the same treatment preceded by three cycles of intensified CHOP (trial HO40). One hundred forty-seven patients were included, 117 of whom had DLBCL. Both studies were carried out in accordance with the modified Declaration of Helsinki. The institutional boards of each participating institution approved both study protocols and all patients gave written informed consent.

Histology

Tumor blocks or unstained tissue sections from all patients with DLBCL as confirmed by central review[1] were retrieved by the local pathologist. Tumors with any follicular pattern were excluded.

Immunohistochemistry and chromosomal breakpoints

IHC was performed on formalin-fixed, paraffin-embedded tissue sections. The following markers were used: bcl2 (clone 124, dilution 1:25, DAKO, Glostrup, Denmark), bcl6 (clone PG-B6p, dilution 1:20, DAKO), CD10 (clone 56C6, dilution 1:20, Novocastra, Newcastle on Tyne, UK), CD21 (clone IF8, dilution 1:10, DAKO), Ki-67 (clone MIB-1, dilution 1:100, DAKO), and MUM1/IRF4 (clone MUM1p, dilution 1:25, DAKO). Antigen heat retrieval in 50 mmol/L TRIS-HCL/2 mmol/L EDTA buffer, pH=9.0 in a microwave oven for 30 minutes was performed for all markers except CD21. For CD21, a pepsin-based antigen retrieval method was used. All immunostains were performed on the Ventana Nexus IHC staining module (Ventana, Tucson, AZ) in accordance with the manufacturer's instructions.

Slides were evaluated semi-quantitatively by two or three independent observers (E.G.B., P.M.K., and G.W.v.I.) and were grouped as follows: for bcl2, tumors with 0% to 10%, 11% to 50%, and 51% to 100% positive tumor cells; for MUM1/IRF4, 0% to 30%, 31% to 50%, 51% to 70%, and 71% to 100% positive tumor cells; for Ki-67, 0% to 50%, 51% to 90%, and 91% to 100%. Bcl6 and CD10 were considered positive when more than 30% of the tumor cells were positive. CD21 was used for the staining of follicular dendritic cell (FDC) networks within the tumor fields using a visual score of 0, +, ++, or +++; staining of tumor cells was ignored.

Classification of GCB versus non-GCB was based on the algorithm of Hans et al,[10] using CD10, bcl6, and MUM1/IRF4 expression with 30% cutoff values for the number of positive tumor cells (Figure 1).

For the detection of chromosomal breakpoints in *MYC*/8q24, *BCL2*/18q21, and *BCL6*/3q27, all cases were studied by segregation fluorescent in situ hybridization (FISH) on paraffin tissue sections in accordance with recently published methods of our group.[17]

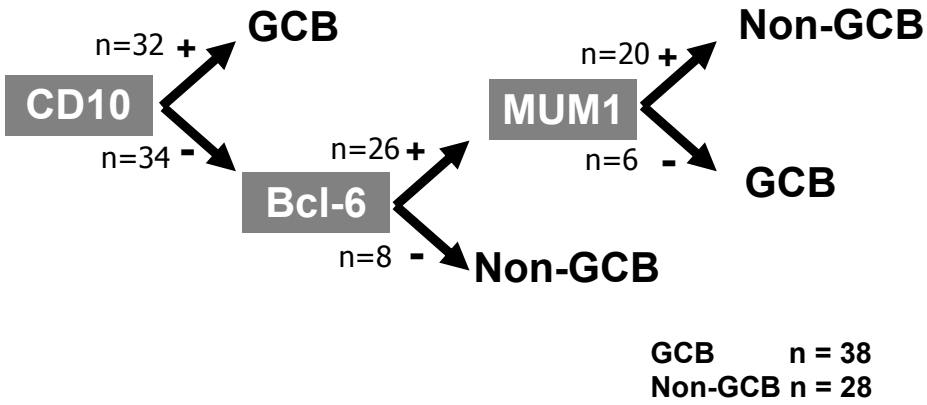

Figure 1. GCB versus non-GCB IHC algorithm.
Distribution of germinal center B-cell (GCB) and non-GCB diffuse large B-cell lymphoma according to the Hans et al algorithm, [10] with cutoff values of 30% for CD10, bcl6, and MUM1/IRF4.

Study end points and statistical considerations

End points of interest were complete response (CR) rate, event-free survival (EFS), disease-free survival (DFS) from CR, and overall survival (OS).[16] Clinical data were retrieved from the HOVON database and analyzed as of November 2005. For comparison of marker distribution between groups, the Fisher's exact test was used. For CR rates between two groups, logistic regression analysis was used. An odds ratio (OR) was calculated with 95% Confidence intervals (CI). EFS, DFS, and OS were estimated using the Kaplan-Meier method, and 95% CI were constructed. Subgroups were compared using the log-rank test. Survival analyses were performed using Cox regression analysis. The hazard ratios (HRs) and corresponding 95% CI were determined for survival end points. Because patients treated in trial HO40 had a significantly better outcome compared with patients in trial HO27, logistic and Cox regression analyses were also performed with adjustment for the trial (HO27 vs HO40). All p-values were two-sided, without adjustment for multiple testing; a level of $\alpha < 0.05$ was considered significant.

RESULTS

Clinical characteristics

Between 1994 and 2001, 147 patients were included in trials HO27 and HO40. Of 117 patients with DLBCL, 73 tissue samples were retrieved, ultimately retrieving adequate tissue from 66 patients (56%), of which 58% were from DLBCL patients from trial HO27 and 55% from HO40. Clinical characteristics of the patients are listed in Table 1. All patients had advanced disease with highly elevated LDH levels and age-adjusted IPI scores ≥ 2. Median follow-up of the 24 patients still alive (seven patients in HO27 and 17 in HO40) was 59 months (range, 23 to 132).

Distribution of markers

IHC and FISH results were assessable in almost all cases (Table 2). For the IHC markers, the percentages of positive tumors were similar in both trials. Slightly more *MYC* and *BCL2* breakpoints were observed in the HO27 study and more *BCL6* breakpoints were observed in the HO40 study. However, absolute numbers were small.

Table 1. Patient characteristics.

	HO27 (n=29)		HO40 (n=37)		Total (n=66)	
	N	%	N	%	N	%
Male	16	55	22	59	38	58
Age, years						
Median	45		52		50	
Range	15-65		18-65		15-65	
Ann Arbor stage						
III	6	21	10	27	16	24
IV	23	79	27	73	50	76
Bulky (>10 cm)	14	48	17	46	31	47
Serum LDH x ULN						
Median	2.8		3.0		3.0	
Range	0.9-28.4		1.5-18.0		0.9-28.4	
Bone marrow involvement	10	34	10	27	20	30
Extranodal localizations >1	15	52	9	24	24	36
Performance score WHO 2-3	14	48	7	19	21	32
Age-adjusted IPI						
2	15	52	30	81	45	68
3	14	48	7	19	21	32
Treatment protocol						
HOVON 27	29	100	-	-	29	44
HOVON 40	-	-	37	100	37	56

Abbreviations: LDH, lactate dehydrogenase; ULN, upper limit of normal; IPI, International Prognostic Index; HOVON, Dutch Belgian Hemato-Oncology Cooperative Group.

Table 2. Distribution of protein expression and molecular breakpoints according to protocol inclusion.

	HO 27 (n=29)		HO40 (n=37)		Total (n=66)	
	N	%	N	%	N	%
Immunohistochemistry						
CD10 > 30%	15	52	17	46	32	48
bcl6 > 30%	26	90	30	81	56	85
MUM1						
0-30%	12	41	16	43	28	42
31-70%	10	34	9	24	19	29
71-100%	5	17	12	32	17	26
No data	2	7	-	-	2	3
GCB type	18	62	20	54	38	58
Ki-67 > 90% (n=65)*	13	46	13	35	26	40
bcl2						
0-10%	11	38	8	22	19	29
11-50%	4	14	7	19	11	17
51-100%	14	48	21	57	35	53
No data	-	-	1	3	1	2
CD21+ FDC (n=58)*	6	25	11	32	17	29
Chromosomal Breakpoints						
MYC/8q24 (n=59)*	6	22	3	9	9	15
BCL2/18q21 (n=59)*	8	30	2	6	10	17
BCL6/3q27 (n=58)*	3	12	12	38	15	26

Abbreviations: GCB, germinal center B-cell; FDC, follicular dendritic cells.
* number of cases tested, percentage is calculated from the number of cases tested.

GCB versus non-GCB phenotype algorithm

Applying the IHC algorithm described by Hans et al,[10] with cutoff values at 30% of positive tumor cells, 38 patients had GCB and 28 patients had non-GCB DLBCL (Figure 1). The majority of GCB cases (84%) were classified as such based on CD10 positivity. Only six CD10-negative cases were classified as GCB-type, based on a combination of bcl6 positivity and MUM1 negativity. In 11 (34%) and four (13%) of the 32 CD10+ cases, MUM1 was positive at 30% and 70% cutoff levels, respectively. Eight of the 34 CD10-negative cases (24%) were bcl6 negative, whereas 20 tumors (59%) were both bcl6 and MUM1 more than 30% positive; both were classified as non-GCB.

Although slightly more patients with GCB tumors had bulky disease, no significant differences were observed in clinical characteristics or treatment according to trial between GCB and non-GCB tumors (Table 3).

Expression of other proteins and chromosomal breakpoints

The distribution of the other IHC markers and chromosomal breakpoints according to GCB versus non-GCB phenotype are listed in Table 4. Bcl2 protein overexpression may be the result of *BCL2* rearrangement or other mechanisms,[10,18-20] and as such confer a different

Table 3. Clinical characteristics and treatment of GCB and non-GCB DLBCL.

	GCB (n=38)		Non-GCB (n=28)	
	N	%	N	%
Male	23	61	15	54
Age, years				
Median	48		52	
Range	(15-65)		(21-65)	
Stage IV	29	76	21	75
Bulky disease	22	58	9	32
LDH x ULN median	3.1		2.9	
BM involvement	13	34	7	25
Extranodal localizations > 1	15	39	9	32
WHO performance status > 1	12	32	9	32
Age-adjusted IPI 3	12	32	9	32
HOVON 40 treatment	20	53	17	61

Abbreviations: GCB, germinal center B-cell; DLBCL, diffuse large B-cell lymphoma; LDH, lactate dehydrogenase; ULN, upper limit of normal; BM, bone marrow; IPI, International Prognostic Index.

Table 4. Chromosomal breakpoints and expression of bcl2 protein, FDC networks, and Ki-67 in GCB and non-GCB Immunophenotype DLBCL.

	GCB (n=38)		Non-GCB (n=28)		*p*
	N	%	N	%	
BCL2 breakpoint (n=58)*					.003
BCL2 break pos / expression pos†	8	25	2	8	
BCL2 break neg / expression pos	10	31	20	77	
BCL2 break neg / expression neg	14	44	4	15	
MYC breakpoint (n=59)*	6	18	3	12	.72
MYC breakpoint only	3	9	2	8	
+ additional *BCL2* breakpoint	3	9			
+ additional *BCL6* breakpoint			1	4	
BCL6 breakpoint (n=58)*	6	18	9	36	.14
CD21 FDC (n=58)*	9	27	8	32	.78
+	5	15	4	16	
++	3	9	2	8	
+++	1	3	2	8	
Ki-67 > 90% (n=64)	17	46	9	32	.31

Abbreviations: FDC, follicular dendritic cells; GCB, germinal center B-cell; DLBCL, diffuse large B-cell lymphoma; pos, positive; neg, negative.

* number of cases tested, percentage is calculated from the number of cases tested; † bcl2 expression > 10% tumor cells.

Table 5. Prognostic value of individual markers on CR and OS

	Complete response						Overall survival						
	N	% CR	95% CI	OR	95% CI	p	N	No. dead	OS5y, %	95% CI	HR	95% CI	p
Total	64	44	31-57	n.a.			66	42	38	26-50	n.a.		
CD10													
Negative	33	30	16-49	1			34	25	29	15-44	1		
Positive	31	58	39-75	3.18	1.14-8.92	.03	32	17	47	27-64	0.52	0.28-0.96	.04
MUM1													
0-70%	46	48	33-63	1			47	27	45	30-59	1		
>70%	17	29	10-56	0.45	0.14-1.50	.20	17	14	18	4-38	2.16	1.11-4.22	.02
Bcl2													
0-10%	18	56	31-78	1			19	9	63	37-80	1		
>10%	45	38	24-53	0.49	0.16-1.47	.20	46	33	26	13-40	2.33	1.10-4.92	.03
CD21 FDC													
Absent	39	31	17-48	1			41	26	35	21-50	1		
Present	17	71	44-90	5.40	1.55-18.8	.01	17	10	39	16-62	0.80	0.38-1.66	.54
Hans algorithm													
GCB	37	51	34-68	1			38	21	45	28-61	1		
Non-GCB	27	33	17-54	0.47	0.17-1.32	.15	28	21	29	14-46	1.87	1.02-3.45	.04

Abbreviations: CR, complete response; OS, overall survival; OR, odds ratio; HR, hazard ratio; NA, not applicable; FDC, follicular dendritic cells; GCB, germinal center B-cell.

* number of patients with CR or OS data available.

clinical behavior.[21] All 10 *BCL2* breakpoint–positive tumors (17%) expressed bcl2 protein. *BCL2* breakpoints clustered in GCB, whereas breakpoint-negative but bcl2 protein–positive tumors were more frequent in the non-GCB group.

BCL6 and *MYC* breakpoints were detected in 26% and 15%, respectively. Four of the nine MYC-positive cases harbored additional breakpoints: three *BCL2*, and one *BCL6* (Table 4). Apart from incidental copy number gain for *BCL2* (n=2), *MYC* (n=4), and *BCL6* (n=4), two cases with gene amplification of *BCL6* were observed.

FDC networks were present in 17 tumors (26%) and were equally frequent in GCB and non-GCB tumors (Table 4). Five FDC+ cases had bone marrow involvement of lymphoma. None of the three cases that could be analyzed showed discordant morphology. FDC+ cases had breakpoints of *MYC*, *BCL2*, or *BCL6* in zero, four, and four cases, respectively. Slightly more GCB type DLBCL had a Ki-67/MIB-1 proliferation rate of more than 90% (46% v 32%).

Prognostic impact of biologic markers on treatment outcome

Because analyses with adjustment for trial (HO27 vs HO40) did not result in different outcomes, only analyses without adjustment for trial are shown. Classification of GCB versus non-GCB had no significant impact on CR rates (Table 5). CR was significantly higher in CD10-positive than in CD10-negative tumors (CR, 58% v 30%; p=0.03), and was significantly higher in tumors with CD21+ FDC networks (CR, 71% v 31%; p=0.01).

GCB conferred better OS than non-GCB phenotype (p=0.04; Figure 2). CD10 positivity (Figure 3A) and MUM1 less than 70% (Figure 3B; but not MUM1 <30%), and bcl2 protein less than 10% (Figure 3C), were associated with superior OS. By applying MUM1 more than 70% instead of more than 30% as the cutoff level for non-GCB classification in the Hans algorithm, nine non-GCB patients changed to GCB type. The predictive value of survival of non-GCB cases based on this MUM1 more than 70% algorithm (HR, 2.11; 95% CI, 1.12

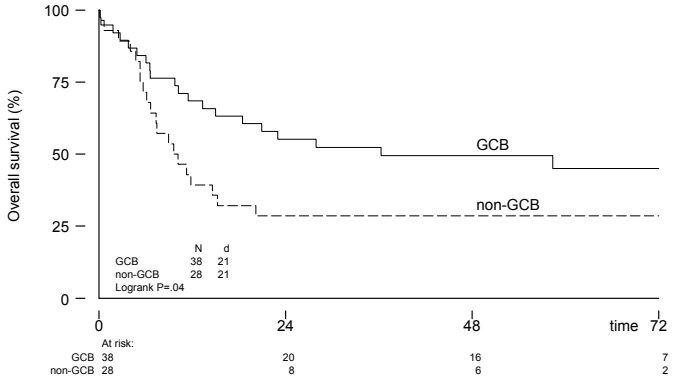

Figure 2. Overall survival of germinal center B-cell (GCB) versus non-GCB immunophenotype.
Abbreviations N, number of patients; d, number of events.

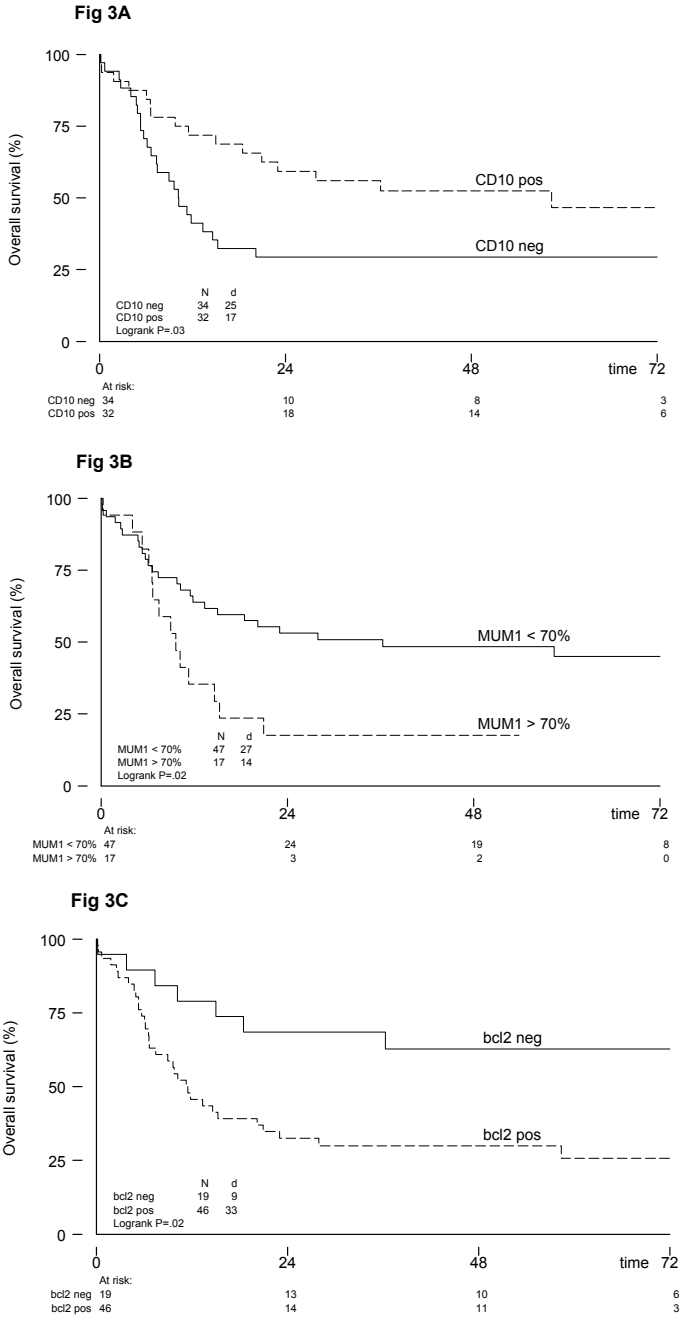

Figure 3. Overall survival according to protein expression of (A) CD10, (B) MUM1/IRF4 more than 70% and (C) bcl2.

(A) CD10 positive (pos) versus negative (neg) diffuse large B-cell lymphoma (DLBCL). **(B)** DLBCL with more than 70% MUM1/IRF4 tumor cells versus cases with less than 70% positive tumor cells. **(C)** bcl2 protein positive (pos; > 10% tumor cells) versus negative (neg) DLBCL. Abbreviations N, number of patients; d, number of events.

to 3.98) might be slightly stronger than the value based on the original algorithm, with a threshold of more than 30% (HR, 1.8; 95% CI, 1.02 to 3.45).

Outcome of bcl2 protein+ GCB cases with (n = 10) or without (n = 8) a *BCL2* breakpoint was not significantly different (median survival, 13 vs 21 months, respectively; p=0.37). Although CR rate was higher in tumors with CD21+ FDC networks, DFS was inferior (p=0.03) and OS was similar (Figure 4). None of the chromosomal breakpoints had a prognostic impact on outcome.

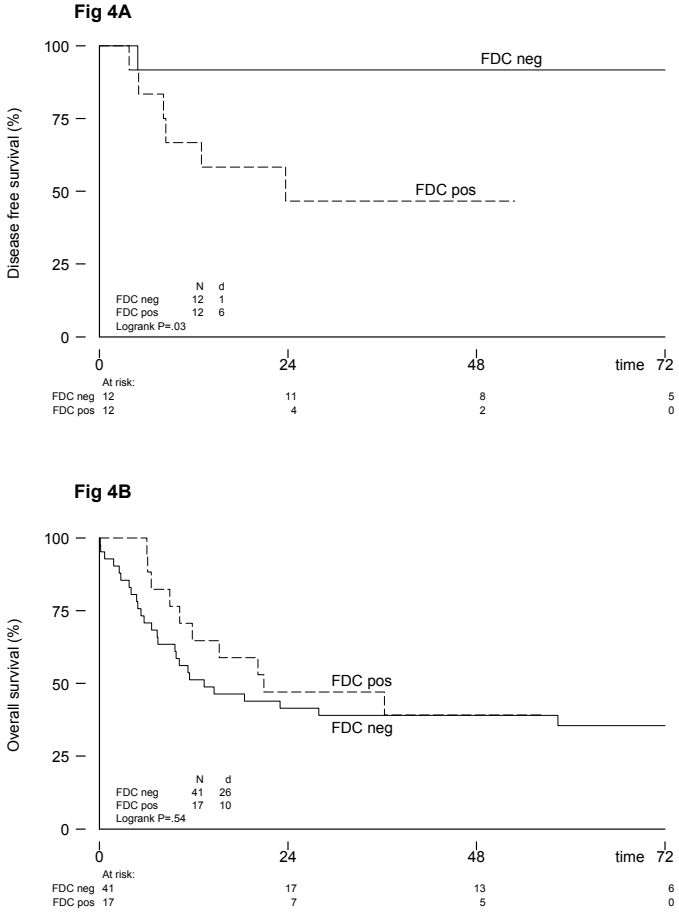

Figure 4. (A) Disease-free and (B) overall survival of follicular dendritic cells (FDC) positive (pos) versus FDC negative (neg) diffuse large B-cell lymphoma.
Abbreviations N, number of patients; d, number of events.

DISCUSSION

We investigated the prognostic relevance of immunophenotype and chromosomal breakpoints in a well-defined group of DLBCL patients with a homogeneous poor-risk IPI score, whose disease was treated up-front with HDT and ASCT. GCB immunophenotype as determined by the Hans algorithm, which includes CD10, bcl6, and MUM1/IRF4 as individual markers,[10] conferred a significantly better survival than non-GCB immunophenotype. Additionally, we found a strong positive association between CD10 expression and response to treatment and survival. Expression of MUM1/IRF4 and bcl2 at cutoff levels of 70% and 10%, respectively, were inversely correlated with survival. Breakpoint analysis of *BCL6*, *BCL2*, and *MYC* had no detectable prognostic impact.

Studies on the clinical relevance of biologic markers in DLBCL are frequently hampered by confounding factors, such as type of treatment and case-mix of patients with different IPI risk scores. This might partially explain why different groups studying the same marker(s) or combinations thereof reported controversial results. In our study, clinical presentation and age-adjusted IPI scores of both cohorts of poor-risk DLBCL patients were almost identical. Treatment, however, was not equal and might have influenced the results; in trial HO40 more chemotherapy (three intensified CHOP courses) had been administered before ASCT. Despite similar CR rates, a significantly better DFS, EFS, and OS in trial HO40 was observed.[16] Nevertheless, the studied tumor markers were almost equally distributed in both trials and all immunophenotypic markers, including GCB- versus non-GCB phenotype, had similar prognostic relevance in both cohorts. Moreover, repeated analyses of prognostic factors with adjustment for trial did not alter the results.

Grouping of DLBCL by gene expression profiling into GCB versus non-GCB derived tumors has originally been based on algorithms containing a myriad of genes.[5] Subsequently, a simple IHC algorithm, based on the expression of only three different proteins (CD10, bcl6, and MUM1/IRF4) could classify tumors in the same groups with similar prognostic results.[10] Our study confirms these findings for survival in a group of poor-risk patients treated with up-front HDT and ASCT.

The prognostic impact of the algorithm not only depends on the relative prognostic value of the individual markers, but also on their hierarchical position in the algorithm and, especially in the case of MUM1/IRF4, on the applied cutoff value. CD10, a marker of follicular center B-cell differentiation,[22] is placed at the first hierarchical level of the algorithm. Reports concerning the prognostic value of CD10 in DLBCL are controversial,[10-12,15,23-26] possibly due to differences in IPI risk factors. In our study of homogeneous age-adjusted IPI poor-risk DLBCL patients, CD10+ immunophenotype correlated most strongly with improved remission rate and outcome, supporting its hierarchical position in the algorithm.

Expression of bcl6, a protein essential for germinal center formation,[27] was observed in 85% of our cases at a cutoff level of 10%, which is in agreement with the literature. All

cases harboring a *BCL6* translocation expressed bcl6 protein, which might be caused by promoter substitution. Additional genetic alterations including point mutations in regulatory sequences of *BCL6* might have accounted for the expression in the many other DLBCL cases in our and other series.[28] The reported prognostic impact of bcl6 protein expression is controversial.[11,12,29] We observed no prognostic impact of bcl6. Furthermore, the contribution of bcl6 in the IHC algorithm on the outcome of classification GCB versus non-GCB immunophenotype in our study was rather small, mainly as a result of the very small percentage of bcl6 negative cases.

Expression of MUM1/IRF4, a marker of terminal differentiation of B cells,[30] is inversely correlated with GCB-type DLBCL in gene expression arrays. MUM1 protein expression is correlated with adverse prognosis in DLBCL according to most,[10,12,31] but not all, reports.[11,15] Cutoff values differ from 20% to 30%[10-12,15] to 80%.[31] In our study, a cutoff value of 30% had no prognostic value. Using a cutoff value of 70% we observed a small group of patients with a very short median survival of 10 months. This effect was independent of CD10 expression. Higher cutoff levels for MUM1 might improve its performance as a single prognostic marker, as well as in prognostic algorithms as suggested by our results.

Recently, Moskowitz et al[32] observed no correlation between GCB versus non-GCB phenotype and outcome in 88 patients with relapsed/primary refractory DLBCL eligible for second-line treatment followed by ASCT. The authors hypothesized that the prognostic value of the IHC algorithm was overcome by HDT and ASCT. In our study of patients treated with HDT and ASCT as first-line treatment, this apparently was not the case. Whether the prognostic value of GCB or other biologic markers in DLBCL would disappear with new treatment modalities remains to be investigated. Notably, neither our patients, nor those of Moskowitz et al, had been treated with anti-CD20 monoclonal antibodies (rituximab).

The results of other markers investigated in our study are of particular interest. We confirmed the strong negative correlation of bcl2 expression with survival, which was reported previously.[3,11,14,31,33-36] We also analyzed the prognostic relevance of bcl2 expression in conjunction with *BCL2*/18q21 breaks, because expression of bcl2 protein might also be the result of distinct other mechanisms and as such confer a different prognosis.[18-21] The negative effect of bcl2 protein expression on survival was observed both in the presence and absence of *BCL2* translocations. Moreover, within the 18 bcl2-expressing GCB-type DLBCL, no difference in outcome was observed between cases with and without the *BCL2* breakpoint. Thus, we could not confirm the negative correlation with survival of *BCL2* breakpoints within the GCB group as reported by Barrans et al.[21] This suggests a dominant effect of overexpression of bcl2 protein, irrespective of the underlying mechanism. Adding rituximab to chemotherapy strongly improves outcome in DLBCL.[37-39] As improvement was most pronounced in patients with tumors expressing bcl2 protein, the negative effect of bcl2 protein expression might diminish in future patients treated with rituximab.[40]

The percentages of DLBCL with breakpoints for *MYC*, *BCL2*, and *BCL6* were comparable with those found in a larger series from a population-based Dutch registry.[3] *MYC* rearrangements in DLBCL may be secondary genetic events and may be associated with aggressive clinical behavior. Half of the *MYC*-positive cases in our study had additional breakpoints of *BCL2* or *BCL6*. Median survival rates for the nine patients with *MYC* rearrangement (including the four cases with double hits) tended to be inferior to rates for patients without the *MYC* rearrangement (nonsignificant). Due to small numbers, our study had insufficient power to detect possibly moderate but clinically relevant differences between the subgroups.

The prognostic impact of CD21+ FDC networks was unexpected. Although not associated with GCB immunophenotype, the clinical data of these cases mimicked the high response rates but increased relapse rates of follicular lymphomas naturally containing such mesh works. Utmost care was taken to exclude lymphomas having any follicular growth pattern or component. In addition, we could exclude discordant lymphoma in three of the five FDC+ cases with bone marrow involvement. Katzenberger et al[41] reported the presence of FDC mesh works in DLBCL with *BCL6*/3q27 translocation. We did not find such a correlation. Nevertheless, the abundance of FDC mesh works in some of our DLBCL cases, even at extranodal sites without naturally occurring FDCs, might indicate an intricate active interaction between the tumor cells and FDC.[41]

In conclusion, in this clinically homogeneous group of poor risk DLBCL patients, we observed a strong prognostic influence of intrinsic biologic markers such as bcl2 expression and GCB immunophenotype, which was not overcome by up-front HDT and ASCT therapy.

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